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MUSCARINIC RECEPTOR SUBTYPES IN THE CNS

by

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I. Introduction

Acetylcholine is a transmitter in both the central and peripheral nervous systems. In a manner similar to many other hormones, the effects of acetylcholine are mediated by cell surface receptors that recognize and bind acetylcholine, subsequently initiating biochemical pathways that lead eventually to the cellular responses. These responses include both excitatory and inhibitory synaptic actions in the CNS (Crawford and Curtis, 1966; Stone, 1972), as well as secretion (Babkin, 1950; Lundquist et al, 1980) and muscle contraction (Parker, 1972; Rang, 1966) in the periphery.

Receptors for acetylcholine are of two major types--nicotinic and muscarinic--differing in location, pharmacology and associated responses (Dale, 1914). The muscarinic receptor, which is found primarily in the CNS and non-skeletal muscle is the subject of this article. The focus is narrowed further in that this review will concentrate on muscarinic receptors in the CNS, mentioning peripheral systems only for the purpose of comparison.

In addition to interneurons in, for example, the caudate nucleus and cerebral cortex, there are a number of known cholinergic pathways in the brain. These include the septo-hippocampal pathway (Raisman, 1966; Mosko et al, 1973), as well as those from the nucleus basalis of Meynert to the frontal cortex (Johnston et al, 1979; 1981) and the pathway coursing through the habenula en route to the interpeduncular nucleus-ventral tegmental area (Fibiger, 1982).

The profound psychotomimetic effects of the potent antimuscarinic

agents in man (Abood and Biel, 1962; Abood, 1968) together with the involvement of cholinergic neurons (Davies and Maloney, 1976; Perry et al, 1977; Bartus et al, 1982), especially those originating in the nucleus basalis of Meynert (Whitehouse et al, 1982) in senile dementia of the Alzheimer type attest to the importance of the central muscarinic system for cognitive function and memory. A decline in the number of muscarinic receptors with aging has also been noted (Perry, 1980; Nordberg and Winblad, 1981). Lesioning studies have demonstrated the involvement of the septo-hippocampal pathway in representational memory employing a task in which rats are trained to alternate between two sources of food in a T-maze (Thomas and Brito, 1980). That muscarinic receptors are involved in the behavioral deficits produced by antimuscarinic agents is bolstered by the correlation between the affinity constants of the anticholinergics for muscarinic receptors and the behavioral disturbances elicited by these agents in animals (Baumgold et al, 1977). Comparable relationships exist for the peripheral systems comparing muscle contraction with affinity constants (Hulme et al, 1978; Yamamura and Snyder, 1974a).

Recent experimental findings in a number of laboratories suggest that muscarinic receptors are heterogeneous. Both agonists and antagonists distinguish between different subclasses of the receptor on the basis of affinity. Whether these subtypes are distinct entities, each linked to separate effector systems or represent different functional or conformational states of the same receptor is a central issue. The evidence for multiple muscarinic receptors and their regulation are discussed in the following sections.

II. Characterization of Muscarinic Receptors

A. Methodology and Criteria for Receptor Binding

These issues have been discussed in detail elsewhere (Burt, 1978b; Cuatrecasas and Hollenberg, 1976), but will be briefly reviewed here because of the major role that binding studies have played in the development of the current view of the muscarinic receptor. It is important to stress that detail must be paid to possible artifacts that may be introduced by the various methods employed. Additionally, the criteria outlined below constitute necessary but not sufficient conditions to conclude that the "binding site" is a bona-fide receptor. Furthermore, each criterion should be reconsidered when features of the assay, such as method, tissue preparation, or labeled ligand are altered. The computer-assisted methods of analysis that are discussed below will be largely unproductive if insufficient attention is paid to these criteria, or to possible artifacts of methodology.

1. Methods

Of the many methods that may be used to measure binding to particulate fractions (see Bennett, 1978), by far the most common are filtration and centrifugation. Filtration is, in many ways, the simpler of the two and places less stringent restrictions on the assay volume. On the other hand, the possibility that the labeled ligand may bind nonspecifically (or, worse yet, "specifically"; cf., Cuatrecasas and Hollenberg, 1976) to the filter itself must be rigorously excluded. Kinetic studies must be carried out to insure that the ligand does not

dissociate appreciably during the washing phase of the assay. Centrifugation assays may yield higher values of nonspecific binding, but seem to represent the better method overall, due to the ease with which the concentration and stability of the unbound ligand can be determined (in the supernatant). Neither labeled nor unlabeled ligands should be degraded or taken up to any significant extent, or bound by soluble proteins or other factors.

In all binding assays, it is necessary to distinguish between specific binding and nonspecific binding. By definition, nonspecific binding is non-saturable and of low affinity, and should, therefore, be proportional to ligand concentration. In practice, nonspecific binding is determined as that which remains in the presence of a concentration of unlabeled ligand which occupies essentially all of the specific sites. In an extensive study of opiate receptor binding, Fischel and Medzihradsky (1981) pointed out possible pitfalls associated with using active and inactive isomers to define specific binding.

2. Criteria

Having determined the total binding and nonspecific binding, the difference between these values is the specific binding. If this difference represents a finite population of receptors, the specific binding should saturate. The affinity of the binding can be estimated initially according to the concentration of ligand that occupies half of the receptors; for successful assays, this value is usually in or below the nanomolar range. More precisely, the affinities of labeled and unlabeled antagonists, as determined by binding assays, should agree with

affinities determined by Schild analysis against responses that are pharmacologically well-characterized. Ideally, comparisons of this type (between binding and response) should be made between assays conducted in identical preparations and under identical conditions, but this is not always possible. Extensive comparisons of the binding and response properties of classical muscarinic antagonists have revealed excellent agreement between the two measures of antagonist affinity (Snyder et al, 1975; Birdsall et al, 1977). Even when identical preparations can be employed, the binding and response properties of agonists may differ, due to possible non-linear coupling between occupancy and response. This feature of agonists can be both disturbing and useful, as discussed in Section IVB. The last criterion in this abbreviated list is that receptors should be found in tissues that exhibit the appropriate pharmacological response(s), but not in tissues that lack such responses. Many studies have demonstrated that the tissues which possess muscarinic receptors according to binding studies are those that respond to muscarinic stimulation.

3. Methods of analysis

Many methods are now available for the analysis of data collected in binding studies, some of which are cited here and below (Weiland and Molinoff, 1981; Molinoff et al, 1981). However, sophisticated computer software cannot in itself assign the model for any set of observations. What it can be used for is to choose between well-defined models, assuming that the data is free of artifact or systematic error. The possibility of such systematic error must often be carefully examined

in separate experiments, the design of which must vary from case to case. The effects of some common artifacts have been described recently on the basis of computer simulations (Munson, 1983). One possible source of systematic error relates to the degree of equilibrium that is achieved during the incubation. For this reason, as well as others, kinetic studies are invaluable. Once on- and off-rates have been determined, the time required to obtain a given degree of equilibrium can be readily estimated (McPherson and Zettner, 1975). It is important to note that the time to equilibrium is markedly dependent on ligand concentration. Further, the presence of competitive inhibitors can alter the time course of the approach to equilibrium (Aranyi, 1980; Ehlert et al, 1981a; Moltusky and Mahan, 1984). Thus, these factors must be taken to account and attainment of equilibrium should be verified empirically. Most paradigms assume that all ligands (labeled and unlabeled) are exposed to the receptor simultaneously; this is especially important when ligands of very high affinity are used (Rodbard et al, 1971).

The most common assumption regarding the binding of ligands to the muscarinic receptor is that of competitive inhibition, and in general, this assumption has been validated. Nonetheless, competitivity cannot be assumed but should be tested directly. The discussion regarding gallamine, below (Section IID), illustrates some of the complexities that may arise. There is now a variety of user-friendly programs available for the analysis of complex competitive interactions (cf., Feldman, 1972; Munson and Rodbard, 1980; DeLean et al, 1982). These programs are commonly used to discern receptor heterogeneity based on direct and indirect binding assays. Subtypes may be defined by the use of selective

labeled ligands or by the competition for the binding of a nonselective labeled ligand by an unlabeled selective ligand. In the muscarinic system, there are no ligands of sufficient selectivity to reliably label a single subpopulation of receptors. It is usually difficult to demonstrate saturability of radiolabeled ligands of moderate selectivity, due to the presence of a low-affinity binding component, therefore, the fulfillment of the criteria for receptor binding (above), depends in part on comparison with indirect binding assays, in which the unlabeled form of the selective ligand competes for the binding of a labeled, nonselective ligand (Birdsall et al, 1978). Furthermore, the binding of nonselective ligands (antagonists) can be correlated more definitively with affinities in response asays, and receptor subtypes are best defined in indirect binding assays in which the labeled ligand is nonselective (Molinoff et al, 1981; Minneman and Molinoff, 1980).

The use of excessively high receptor concentrations may lead to problems of interpretation in both direct and indirect binding assays. When the receptor concentration exceeds one-tenth the dissociation constant of the labeled ligand, the resulting depletion of the free ligand concentration will affect estimates of the affinity of the ligand-receptor interaction (Chang et al, 1975). If the free ligand concentration can be accurately determined, however, true values of affinity will be obtained from direct binding assays (Chang et al, 1975). When indirect binding studies are performed, the observed affinity of the unlabeled ligand is a function of the concentration of both the labeled ligand and the receptor. The effect of the concentration of labeled ligand is readily accounted for (Cheng and Prusoff, 1973), while the

effect of receptor concentration is not (Jacobs et al, 1975). Recently, however, a correction for the effect of receptor concentration has been published (Linden, 1982); computer methods may also correct for the depletion of radioligand (Delean et al, 1982). Investigators should beware of the contribution of nonspecific binding to radioligand concentration; binding to filters, for example, occurs after the assay. In extreme conditions, errors in the specific activity of the radioligand may lead to erroneous estimates of depletion. Estimates of the depletion of high-affinity unlabeled ligands are even more difficult. Finally, similar artifacts may occur when response assays are conducted in small volumes; in some cases, the magnitude of the error involved can be assessed by performing binding assays under identical conditions.

B. Classical Antagonist Binding

The binding of classical muscarinic antagonists, in the CNS and the periphery, satisfies the criteria outlined above (Section IIA) for specific receptor binding. Extensive studies by many laboratories had indicated for many years that these antagonists bind to a single, homogeneous population of receptors with the same affinities that they exhibit in response assays. Thus, the numbers of receptor sites (B_{max}) determined by different labeled antagonists are in good agreement and different unlabeled competitors displace the same amount of binding of a given labeled ligand (Hulme et al, 1978). Antagonists have been found to bind to different regions of the CNS with the same affinities (Ellis and Hoss, 1980; Birdsall et al, 1980) and also bind to central and peripheral receptors with the same affinities, which agree with affinities obtained

from response measurements (Beld et al, 1975; Snyder et al, 1975). Within the CNS, muscarinic receptor densities in a variety of species follow the following pattern: striatum > hippocampus ≅ cerebral cortex > diencephalon ≅ tectum > medulla-pons > cerebellar hemispheres.

In the last few years, it has become apparent that the interactions of antagonists with muscarinic receptors are not as simple as the earlier studies indicated. It is now known that many of the classical antagonists express different affinities in different tissues and that, at least under some conditions, they can be shown to bind heterogeneously within a given tissue. Other (truly selective) antagonists bind heterogeneously both within and among tissues. These deviations from ideal mass-action behavior are discussed below (Section IID). However, it is important to emphasize the fact that under many "normal" conditions the classical antagonists define a single homogeneous population of receptors, within which agonists and the truly selective antagonists define their respective subpopulations of muscarinic receptors. As discussed in Section IIA, the use of a non-selective labeled ligand improves the feasibility of complicated binding assays involving selective unlabeled ligands.

C. Agonist Binding

Under the same conditions that give rise to homogeneous binding curves for antagonists, the binding of agonists deviates from the form of a single site. Hill coefficients for the inhibition of the binding of labeled antagonists by agonists such as carbachol are considerably less than unity throughout the CNS (Birdsall et al, 1978, 1980; Ellis and

Hoss, 1980, 1982). Alternative plots of such data show that log dose-occupancy curves are flattened relative to the mass-action isotherm or that Scatchard plots are concave-up. The binding of two labeled agonists, oxotremorine-M (Birdsall et al, 1978) and cis-methyl-dioxolane (Ehlert et al, 1980a), has been investigated. The binding observed in these studies at the highest ligand concentrations that are technically feasible is, in general, considerably less than that obtained from antagonist binding studies. Also, unlabeled agonists compete with greater affinity for the binding of labeled agonists than for the binding of labeled antagonists. Explanations for the binding characteristics of agonists have included: (1) that the presence of agonists or antagonists shifts the proportion of receptors that are in the "agonist" or "antagonist" state (Snyder, 1975; Snyder and Bennett, 1976); (2) that increasing concentrations of agonist progressively desensitize the receptor (Birdsall et al, 1978); (3) that the interaction of agonists with the receptor is negatively cooperative (ibid); and (4) that there are multiple subpopulations of muscarinic receptors that are discerned by agonists but not by antagonists (ibid). Since the binding curves of agonists are stable over time, model (2) seems unlikely (Birdsall et al, 1978). The strongest support for model (4) over the others has come from studies in which a given proportion of receptors is blocked by irreversible or very slowly reversible antagonists in the presence or absence of agonist. The results of such experiments show that there are preformed subpopulations of receptors that do not interconvert under the conditions of the binding assays. Qualitatively similar results have been obtained for muscarinic receptors derived from forebrain and

brainstem regions (Birdsall et al, 1978; Ellis and Hoss, 1980). These studies illustrate that the choice of experimental design, rather than extensive curve-fitting, can be the key to discrimination between complex models of receptor action.

Thus, the shapes of the binding curves of agonists seem to be due to the presence of subpopulations of receptors which possess different affinities for agonists but homogeneous affinities for antagonists. The observation that agonist IC_{50} values (concentration that inhibits 50% of the binding of labeled antagonist) varied across brain regions (Aronstam et al, 1977, 1978a) suggested that these differences might be due to differing proportions of the subpopulations in the different regions. Analyses of the occupancy curves for agonists found that the binding in different regions of the brain could not be explained as representing different proportions of two subpopulations, and it was concluded that the brain must possess at least three different subpopulations (Ellis and Hoss, 1980). More detailed analysis of the binding of agonists demonstrated that the postulation of a third site significantly improved the agreement between the model and the data (compared to two sites) in the medulla-pons (Birdsall et al, 1980). Further, the same three sites were sufficient to describe the binding of agonists in the other regions. Sokolovsky et al (1983) have pointed out that it is probably not possible at the present time to discriminate between models of great complexity, such as a model of three sites in comparison with a model of two sites plus cooperativity or isomerization. These authors suggested that most studies neither require nor justify the effort involved in substantiating a third site. The development of selective antagonists (Section IID) may

generate fresh approaches to these questions.

D. Non-Uniform Antagonist Binding

Initial reports of heterogeneity in the binding of muscarinic agonists, but not antagonists (Birdsall et al, 1978; Hulme et al, 1978) were cause for both excitement and disappointment. While these data suggested that the many physiological and behavioral effects that are mediated by the muscarinic system might be related to distinct subpopulations, they did not hold much promise for the exploitation of this information. First, receptors are most usefully classified according to the affinities of antagonists, because of the difficulties that can be encountered in relating the binding data and response data of agonists (Burgen, 1979; Swillens and Dumont, 1980; Kenakin, 1983). Second, all of the agonists tested seemed to have the same order of affinities for the subpopulations (Birdsall et al, 1980). This feature led Birdsall et al (1977) to propose that the receptor heterogeneity that is perceived by agonists is due to constraints that the coupling of effectors places on the properties of the activated state of a single receptor. If these constraints did not affect the ground state of the receptor, then the binding of antagonists would not be affected. Within the general guidelines of this theory, however, there are two possibilities for the existence of antagonist selectivities. First, there may be minor variations in the receptive site of the different subpopulations that are overshadowed by coupling constraints in the case of agonists and that are not discerned by classical antagonists, but may be discerned by novel antagonists. Second, the coupling constraints may

induce minor variations in a common receptor site, such that a similar situation prevails. In either event, selective pharmacological manipulation of muscarinic subpopulations might be feasible and it is of considerable importance that various forms of selectivity can now be demonstrated for antagonists.

1. Selectivity between tissues and responses

It has been noted above that years of study with classical muscarinic antagonists had indicated that the muscarinic receptors in different tissues were indistinguishable. In their study of heterogeneous antagonists, Fisher et al (1976) pointed out that the earlier conclusion was the "correct and logical conclusion" from the data that these earlier studies had collected, and they suggested that studies with molecules of greater rigidity and lower affinity would be necessary to detect heterogeneity in the effects of antagonists. The rigid antagonists that this group has synthesized do indeed display widely differing affinities in antagonizing different muscarinic responses in vivo. Molinoff et al (1981) have pointed out that the determination of receptor subtypes by in vivo studies is complicated by problems of access barriers and metabolic effects that may vary from tissue to tissue. However, these reservations do not apply as strongly to comparisons within a tissue. Based on the potencies of these relatively low-affinity, rigid antagonists, Fisher et al (1980a) have concluded that there are subtypes of central muscarinic receptors.

Support for the existence of separate muscarinic receptors has also come from studies of the response properties of antagonists in vitro.

Although a previous report had found little evidence for differences between the muscarinic receptors of ileum, bronchial muscle and iris (Barlow et al, 1972), studies which compared antagonist affinity constants of atrium to those of ileum did find such differences (Barlow et al, 1976, 1980). The authors noted that quaternary salts were more selective than their tertiary analogs. Other examples of tissue and response selectivity will be presented in succeeding sections.

2. Heterogeneous binding properties of classical antagonists

As stated above, under many experimental conditions, especially those in which physiological buffers and salts are included, the equilibrium binding curves of classical antagonists follows the mass-action isotherm. However, recent studies have found the binding of even the classical antagonists to be more complex in several ways. These deviations from the mass-action formulation can be grouped under three separate headings: (1) conditions of low ionic stength, in which equilibrium binding curves suggest heterogeneity of receptors; (2) kinetic studies that indicate that there are multiple conformational states of the receptor-ligand complex (isomerization); and (3) studies that indicate complex cooperative interactions in the binding of antagonists.

Thus, in the striatum, Ehlert et al (1981b) have found the binding of {^{3}H}-QNB to be biphasic when assays were performed in 50 mM sodium HEPES, 10 mM $MgCl_2$, and 10 μM Gpp(NH)p. The additional presence of apormorhine (1 μM) appeared to convert the low affinity sites to high affinity, resulting in a homogeneous binding curve for {^{3}H}-QNB. A

somewhat similar phenomenon is observed in the heart, where the binding of {^{3}H}-NMS suggests the presence of two populations of receptors when assays are conducted in 20 mM HEPES, in the absence of of inorganic ions (Hulme et al, 1981a). The inclusion of 100 μM GppNHp converts the binding curve to that expected of a single, homogeneous, high-affinity site. The fact that these results are obtained in the absence of added Mg^{2+} may be related to the observation that added Mg^{2+} is also not necessary for the modulation of agonist binding by guanyl nucleotides in heart tissue (Hulme et al, 1981b). A difference in the level of endogenous membrane-bound Mg^{2+} may explain these differences and also be responsible for the opposite effects of low ionic strength on antagonist binding in the heart and cerebral cortex. In contrast to the effects described above for cardiac receptors, the affinity of the cortical receptor for antagonists is increased in low ionic strength buffers, and homogeneity of binding is preserved (Birdsall et al, 1979b).

There are now a large number of reports to indicate that the binding of muscarinic antagonists involves an isomerization step, although binding at equilibrium is entirely in accord with mass-action kinetics. These findings are not paradoxical, as the properties of binding under equilibrium conditions are independent of the number of conformational states (Prinz, 1983). Support for the concept of isomerization then comes necessarily from detailed kinetic studies of on- and off-rates. Studies in many different tissues and with several different labeled ligands have found that dissociation of labeled antagonists from the receptor is not a monoexponential process, and that the dissociation curves change qualitatively, depending on the preceding time of

association (Galper et al, 1977; Galper and Smith, 1978; Klein, 1980; Schimerlik and Searles, 1980; Kloog and Sokolovsky, 1978a,b; Jarv et al, 1979). Other studies have found that the association kinetics of antagonists are not compatible with a simple bimolecular process or that equilibrium dissociation constants do not agree with the ratio k_{-1}/k_1 (see Sokolovsky et al, 1983). Similar findings concerning the binding of the β-adrenergic antagonist {^{125}I}-IHYP (iodohydroxybenzylpindolol) have led to a similar model of receptor isomerization (Ross et al, 1977). Burgisser et al (1981) noted that many of these studies involved the use of racemic mixtures of labeled ligands. They went on to show that the presence of the inactive form of the radiolabeled ligand can give rise to artifactually complex binding phenomena. Tolkovsky (1982) has examined a similar kinetics problem concerning the binding of {^{3}H}-etorphine to membranes from sheep caudate nucleus. He suggested that two independent sites were responsible, but also discussed the additional complexities introduced by the use of a racemic labeled ligand. Thus, while the bulk of evidence supports the possibility of isomerization in the binding of muscarinic antagonists, some past experiments might bear reinvestigation with the purified isomers (e.g., (-)-{^{3}H}-1-QNB) that are now available.

Several studies have reported that, even under equilibrium conditions, the binding of classical muscarinic antagonists differs from the simple mass-action isotherm in buffers that approximate physiological ionic composition. On the basis of binding studies with several antagonists, Henis and Sokolovsky (1983) concluded that there are negatively cooperative interactions between muscarinic receptors in the adenohypophysis of the rat, but not in the medulla-pons or cerebral

cortex. On the other hand, Kloog and Sokolovsky (1978a) found curvilinear Scatchard plots for the binding of {^{3}H}-scopolamine and {^{3}H}-4NMPB to homogenates of whole mouse brain, which indicate either negative cooperativity or the presence of heterogeneous sites (Sokolovsky et al, 1983). Hedlund et al (1980, 1982) have recently reported very complex patterns of binding for the classical muscarinic antagonist QNB and 4NMPB in rat cortex. Plots of binding vs. concentration of labeled ligand show two plateaus, a finding which requires the assumption of either positive cooperativity or complex interactions of negative and positive cooperativity (see Hedlund et al, 1982). It has been noted that the optimal buffer for demonstrating this phenomenon is Krebs buffer containing 5 mM HEPES (Hedlund et al, 1982). Markedly different results were obtained with 50 mM phosphate buffer, although the ionic strengths of the two buffers were equal (Hedlund, 1981).

3. Selective antagonists

In 1980, Hammer et al reported that the tricyclic compound, pirenzepine, displayed heterogeneous binding profiles within and among tissues, under conditions in which classical antagonists such as {^{3}H}-NMS bind homogeneously. Pirenzepine had previously been shown to antagonize muscarinic responses with widely varying affinities, depending on the location of the receptor (see Hammer, 1982); but the additional finding of heterogeneous binding underlined its importance as a prototypical selective antagonist. Pirenzepine binds with highest affinity in the forebrain, and with lesser affinities in the brainstem, heart and ileum (Hammer et al, 1980). The recent availability of {^{3}H}-pirenzepine

(Watson et al, 1982) adds another useful tool to the armament of receptorologists, but its relatively low affinity and selectivity may limit its utility (Section IIA).

Gallamine is a neuromuscular blocking agent that has been found to express antimuscarinic effects (Clark and Mitchelson, 1976; Bird and Aghajanian, 1976; Rathbun and Hamilton, 1970). In an attempt to screen selective muscarinic antagonists, we chose to investigate such unusual antimuscarinics. We agreed with the reasoning of Fisher et al (1976) that drugs of greatest selectivity would have relatively low affinities, and felt that investigation of well-known antagonists would prove less fruitful. Gallamine competed more potently for the binding of ^{3}H-QNB in the brainstem than in the forebrain, in a manner that suggested heterogeneity of binding sites in both regions (Ellis and Hoss, 1982). When sites that had low affinity for carbachol were selectively eliminated, the affinities of both gallamine and carbachol were increased, suggesting that the eliminated sites also had low affinity for gallamine. Furthermore, in the brainstem, the sites that were left after this treatment appeared to possess homogeneous affinity for gallamine, although not for carbachol (Ellis and Hoss, 1982). These findings, and the observation that $\{^{3}H\}$-QNB could completely overcome the inhibition by gallamine, led us to conclude that the interaction between gallamine and $\{^{3}H\}$-QNB was competitive.

A subsequent report by Stockton et al (1983) presented convincing evidence, especially kinetic evidence, that gallamine's interaction with $\{^{3}H\}$-NMS is not competitive. The contrast between these two reports led us to compare the effects of gallamine on the binding properties of $\{^{3}H\}$-

NMS and {^{3}H}-QNB in a single system (Ellis and Lenox, in preparation). It can be seen in Table 1 that we were able to replicate the results of Stockton et al (1983) when {^{3}H}-NMS was the labeled ligand. That is, the presence of 100μ m gallamine dramatically slows the rate of dissociation of {^{3}H}-NMS from the receptor; also, concentrations as low as 3 μ m slow the rate of association of {^{3}H}-NMS with the receptor approximately ten fold. At the same time, similar kinetic studies in the same membrane preparations did not find evidence of non-competitive inhibition of the binding of {^{3}H}QNB. At concentrations of 15 μM and 100 μM, gallamine did not significantly alter the kinetics of association or dissocation of {^{3}H}-QNB. Therefore, we do not feel that noncompetitive interactions obscured the previously reported studies in which {^{3}H}-QNB was employed as the labeled ligand (Ellis and Hoss, 1982). One possible explanation for the data presented in Table 1 is that gallamine does bind allosterically, but that the binding of gallamine or QNB reduces the affinity of the other to such an extent that binary complexes cannot be demonstrated. Such an interaction would be indistinguishable from true competivity. Another possible explanation is that gallamine may bind to an allosteric site as well as to the site to which classical antagonists bind. According to this scheme, QNB must be insensitive to the same allosteric interaction that affects the binding of NMS. It is not unlikely that the positively charged NMS might interact with the receptor in a different manner than does the very lipohilic QNB. Dunlap and Brown (1983) have suggested that gallamine interacts both competitively and allosterically with cardiac muscarinic receptors. Evaluation of this and other possibilities must await future studies.

The atypical agonist, McN-A-343 (3-(M-chorophenyl-carbamoyloxy)-2-butynyltrimethyl ammonium), raises blood pressure and heart rate when applied systemically, apparently by stimulating ganglionic receptors with greater affinity than it expresses in heart or smooth muscle (Roszkowski, 1961; Hammer and Giachetti, 1982). It has been suggested that McN-A-343 may bind to the same allosteric site to which gallamine appears to bind in the heart, although it displaces the binding of {^{3}H}-NMS to cortical receptors by a competitive mechanism (Birdsall et al, 1983c). It is somewhat surprising that guanyl nucleotides regulate the binding of McN-A-343 in the heart, as they do that of typical, presumably competitive agonists (Birdsall et al, 1983c). It is also interesting that McN-A-343 has higher affinity for L sites than for H sites in the cortex (Birdsall and Hulme, 1983), while gallamine seems to possess a similar order of affinities to that of typical agonists (Ellis and Hoss, 1982).

Despite the controversy over the nature of gallamine's interaction with the muscarinic receptor, there is agreement that it distinguishes receptor subtypes (Ellis and Hoss, 1982; Birdsall and Hulme, 1983; Birdsall et al, 1984; Dunlap and Brown, 1983). In view of the paucity of antagonists that distinguish subpopulations of muscarinic receptors, gallamine will undoubtedly prove to be a useful tool in future characterizations of the muscarinic receptor.

E. Sulfhydryl and disulfide reagents

Sulfhydryl alkylating reagents such as N-ethylmaleimide (NEM) and membrane oxidizing reagents such as 5,5'-dithiobis (2-nitro-benzoic acid) (DTNB) and potassium ferricyanide increase the affinity of agonists for

the muscarinic receptor (Aronstam et al, 1978a; Aronstam and Eldefrawi, 1979a). The effect of NEM is seen as an increase in the number of high affinity receptors (Aronstam, et al, 1977). The presence of agonists but not antagonists increases the ability of NEM to enhance agonist binding, indicating that agonist binding results in the exposure of sequestered sulfhydryl groups. On the other hand, reducing agents, such as dithioithreitol (DTT) or 2-mercaptoethanol decrease agonist affinity (Aronstam and Eldefrawi, 1979a). The effects of oxidizing and reducing agents appear, moreover, to be reversible (Aronstam and Eldefrawi, 1979a; Hedlund and Bartfai, 1979). The effects on agonist binding can be observed at concentrations of the alkylating, oxidizing or reducing reagents that have no effects on antagonist binding. In contrast, NEM decreased the affinity of oxotremorine in cardiac membranes (Harden et al, 1982).

The sulfhydryl reagent PCMB inhibits both antagonist and agonist binding (Aronstam, et al, 1978a). Pretreatment with NEM protects against the inhibition of antagonist but not agonist binding, suggesting the interaction of at least two different sulfhydryl groups with the muscarinic receptor. Recently, these findings have been corroborated and extended by a detailed study of the effects of PCMB on muscarinic receptors in the rat cerebral cortex (Birdsall et al, 1983a,b).

In conclusion, these studies suggest that the state of membrane sulfhydryl and disulfide moieties may regulate muscarinic receptors. As discussed in detail below (Section VC), Cu may regulate muscarinic receptors in vivo by binding to membrane sulfhydryl groups.

F. Metals and GTP

Transition and heavy metals affect muscarinic receptors by inhibiting the binding of antagonists at higher concentrations (Aronstam et al, 1978a; Aronstam and Eldefrawi, 1979b) and by increasing the binding of agonists at lower concentrations that do not affect antagonist binding (Aronstam et al, 1978a). The effect on antagonist binding is reversible and competitive, with Hg^{2+} having the greatest inhibitory potency (ID_{50} = 10^{-7} M). Other metals including Cu^{2+}, Fe^{2+}, and Pb^{2+} were much less effective, having ID_{50} values between 10^{-5} and 10^{-4} M. Increasing the availability of sulfhydryl groups did not affect the inhibition of antagonist binding by heavy metals. Thus, the inhibitory effects of metals at high concentrations seem to be due to a direct interaction with the receptor that does not involve sulfhydryl groups. It is conceivable that inhibition of muscarinic cholinergic receptors may be one of the toxic effects of metals such as Hg^{2+} and Pb^{2+}.

At lower concentrations, metals such as Cu^{2+}, Cd^{2+}, Pb^{2+} and Zn^{2+} increased agonist binding without affecting antagonist binding (Aronstam et al, 1978a). Since there was no further increase in agonist binding after pretreatment with NEM, sulfhydryl groups are probably involved in the ability of lower concentrations of metals to increase agonist binding.

There is a striking difference between the effect of Cu^{2+} on forebrain and brainstem receptors (Farrar and Hoss, 1984). For example, 5 μM Cu^{2+} significantly inhibits QNB binding in the forebrain, but has almost no effect on the brainstem. Further, as shown in Fig. 1, 3μ M Cu greatly increases CCh binding in the forebrain but not brainstem. Thus,

Cu distinguishes between forebrain and brainstem receptors in vitro. The inclusion of 1 μM Cu^{2+}, which had no effect on QNB binding, increased the fraction of sites with high affinity for carbachol from 42 to 70% according to a two-site fit in forebrain (Farrar and Hoss, 1984). Thus, low concentrations of Cu^{2+} can affect the distribution between high and low affinity agonist receptors in vitro. The effects of Cu^{2+} on agonist binding were reversible with triethylenetetramine (Farrar and Hoss, 1984), suggesting that the effects were not owing to some Cu-related oxidative or catalytic process. In contrast to the effects of Cu and other transition metals, the effects of alkali and alkaline earth metals appear to be absent or weak (Birdsall et al, 1979b).

Guanine nucleotide in the presence of Mg^{2+} decrease the binding of agonists but not antagonists to muscarinic receptors (Sokolovsky et al, 1980), in brainstem but not in forebrain regions of mouse. In that study, GTP induced an apparent interconversion between high and low affinity receptors. In a subsequent report, Gurwitz and Sokolovsky (1980) demonstrated that micromolar concentrations of GTP could reverse the increase in agonist affinity induced by 1 mM Mn^{2+}, Ni^{2+} or Co^{2+}.

The GTP effect on agonist binding, which is weaker in brain than in heart (Berrie et al, 1979; Ehlert et al, 1980b), is presumably a reflection of the activity of the regulatory protein complex Ni, which binds GTP and mediates the coupling between receptors and the inhibition of adenylate cyclase (Jakobs, 1979). The hydrolysis of GTP terminates the coupling between receptor occupancy and adenylate cyclase. Muscarinic receptor-induced inhibition of adenylate cyclase has been demonstrated in several tissues including NG 108-15 cells (Nathanson et

al, 1978) and rabbit heart (Jakobs et al, 1979) in addition to rat brain (Olianas et al, 1983). In brain, the effect requires GTP. The muscarinic receptor-induced inhibition of adenylate cyclase is discussed in detail in Section IIIB.

G. Affinity Labeling

Affinity labeling of muscarinic receptors in brain has been accomplished by using nanomolar concentrations of tritiated propylbenzilylcholine mustard. Specificity is demonstrated by the ability of 1 μM atropine to inhibit the labeling. Subsequent solubilization and electrophoresis has given molecular weights of approximately 80,000 daltons for guinea pig, rat and frog brains (Birdsall et al, 1979a) or 75,000 daltons for bovine caudate membranes (Ruess and Lieflander, 1979).

More recently, Amitai and collaborators (1982) have synthesized two photoaffinity labels for muscarinic receptors based on the incorporation of an azido group into one of the phenyl rings of 3-quinuclidinyl benzilate and N-methyl-4-piperidyl benzilate (NMPB). The tritiated probes bound in a potent, specific and reversible manner in the dark. Photolysis in the presence of nanomolar concentrations of azido-{^{3}H}-N-methyl-4-piperidyl benzilate (azido{^{3}H}-NMPB) produced an irreversible loss of receptors that was inhibited by atropine. Gel electrophoresis showed that the label was associated with a single protein of molecular weight 86,000 daltons in the rat cerebral cortex.

In a subsequent report (Avissar et al, 1983), the use of the azido-{^{3}H}-NMPB probe showed that, in brainstem areas, specific binding occurs

to a 180,000 dalton protein in addition to the 86,000 dalton species. Further, both proteins can be dissociated to 40,000 dalton peptides by treatments that cleave ester bonds. Based on these findings, they propose that muscarinic receptors can exist as a 80,000 dalton dimer of two 40,000 dalton subunits joined by covalent bonds or a tetramer of 160,000 daltons comprised of two dimers; and further, that the dimer corresponds to the low and the tetramer the high agonist affinity state of the receptor. It is also possible that the higher molecular weight form involves the receptor associated with some other protein. It is of interest in this regard that agonists can induce the association between receptor and guanine nucleotide regulatory protein (see, for example Smith and Limbird, 1981; Kilpatrick and Caron, 1983).

III. Responses Elicited by Muscarinic Activation

As mentioned in the Introduction, muscarinic systems mediate a wide variety of physiological and behavioral responses. This section, however, will be restricted to biochemical responses, with minor exceptions. By this, we mean those responses that can be demonstrated in vitro in tissue slices, cultured cells, or subcellular fractions. These responses are more compatible with the biochemical portions of this review that deal with receptor binding. More to the point, these responses are more readily related to binding parameters because they can be carried out under conditions that can also be used in binding studies. This advantage will be discussed below and in a subsequent section.

A. Turnover of phosphatidylinositol

Stimulation of the turnover of phosphatidylinositol (PhI) can be elicited by a wide range of hormones in an equally large variety of physiological systems, including many regions of the brain (Michell and Kirk, 1981). On the basis of the close association of PhI turnover and calcium mobilization, coupled with the lack of calcium-dependence in the PhI response (but see below), Michell (1975) has suggested that the turnover of PhI may be the stimulus that leads to a rise in intracellular Ca^{2+} levels. Several mechanisms have been proposed to explain the apparent link between PhI metabolism and calcium mobilization. Most generally, the hydrolysis of PhI may result in local changes in membrane structure that lead to the opening of a calcium channel (Jones et al, 1982). A more specific version of this theory suggests that PhI 4,5-biphosphate ($PhIP_2$), which has high affinity for Ca^{2+}, may project into the calcium channel to bind and hold Ca^{2+}. Cleavage of the inositol triphosphate would then allow an unimpeded flow of Ca^{2+} into the cell (Jones et al, 1982). Another suggestion is that phosphatidic acid (PhA), regenerated from the 1,2-diacylglycerol that is produced by the hydrolysis of PhI, may function as a Ca^{2+} ionophore (Putney et al, 1980). The potency of PhA in partitioning calcium into chloroform from aqueous solutions is roughly equal to that of the calcium ionophore A23187 (ibid). Addition of exogeneous PhA stimulates several responses that are associated with PhI turnover/calcium flux (Putney et al, 1980; Salmon and Honeyman, 1980). The recently discovered phospholipid-dependent protein kinase (C-kinase; Nishizuka, 1983) represents another possible transduction mechanism, since its activity is enhanced by 1,2-

diacylglycerol. The diacylglycerol which results from cleavage of the head group of PhI may be the endogenous activator, since the presence of the fatty acids common to PhI confer the greatest activity. Activation of this cAMP-independent kinase could lead to the phosphorylation of membrane proteins and, consequently, to alterations in membrane permeability.

Whether the association between PhI turnover and Ca^{2+} mobilization is as general as originally suggested (Michell, 1975) is a matter of some controversy (see Hawthorne, 1982; Michell, 1982; Cockcroft, 1981). Thus, in some systems the turnover of PhI is dependent to some extent on extracellular Ca^{2+} (see Cockcroft et al, 1980; Cockcroft, 1981). For example, the acetylcholine-induced PhI effect in synaptosomes requires micromolar levels of extracellular Ca^{2+} (Griffin et al, 1979), while the muscarinic stimulation of the formation of cGMP in brain slices requires Ca^{2+} in the millimolar range (Hanley and Iversen, 1978). In pineal cells (Smith and Hauser, 1981), the adrenergic stimulation of PhI effect requires Ca^{2+} only in the absence of 1 mM inositol. This suggests that a requirement for calcium may indicate the metabolic needs of the cell rather than a direct interaction of calcium in the initiation of PhI turnover. The concentrations of PhI, PhIP and $PhIP_2$ are determined by complex metabolic pathways (Jones et al, 1982) which may be sensitive to drastic fluctuations in Ca^{2+} levels. Therefore, while the finding that PhI turnover is independent of external Ca^{2+} represents strong evidence against a role for Ca^{2+} in the response, the converse finding is not so conclusive. Vasopressin has recently been reported to stimulate degradation of PhI in purified liver plasma membranes (Wallace et al,

1982). The use of such simplified systems may provide a more definitive answer to the question of precedence in PhI turnover/Ca^{2+} mobilization.

Whatever the mechanism, muscarinic agonists stimulate the labelling and/or breakdown of PhI in many systems, including sympathetic ganglia, parotid gland, heart, smooth muscle, and brain (Michell and Kirk, 1981). Table 2 demonstrates the muscarinic nature of the cholinergic stimulation of the turnover of PhI in slices of rat forebrain. The maximal stimulatory effect is approximately 100% and is blocked by scopolamine. Other studies have indicated that a stimulation of about 50% can be elicited in slices derived from the tectum (not shown). The dose-response curve for the stimulation elicited by carbachol is shown in Fig. 2, along with the occupancy curve for carbachol, inferred from competition vs {^{3}H}-NMS. Several features of Fig. 2 deserve comment. The occupancy curve for carbachol is markedly flattened, as in membranes, while the PhI response can be adequately fitted by a one-site curve. The curve for the PhI response does not agree with either K_H or K_L (derived from a two-site fit to the carbachol binding curve). The affinity of carbachol is much lower than the value commonly found in membrane studies, as previously reported by Gilbert et al (1979). Comparisons of binding and response curves of agonists are fraught with difficulties (see section IVA). However, we have detected neither desensitization nor a receptor reserve in studies to date and tentatively suggest that the PhI response of brain slices may be associated with a single population of muscarinic receptors, having an affinity between the values of K_H and K_L obtained by a two-site fit. This site may be related to the "H" site of the three-site fit of Birdsall et al (1980).

Fisher et al (1983) have recently reported similar comparisons of binding and response in synaptosomal preparations derived from cerebral cortex. In this synaptosomal study, the binding of carbachol occurred with higher affinity and the response with lower affinity, compared to our data in slices (Fig. 2). The response curve agreed well with the law of mass action, with an affinity very near to that of the low affinity site (approximately 100 μM) derived from a two-site fit of the binding data. Bethanacol, pilocarpine, arecoline, and oxotremorine were found to be partial agonists of low efficacy in the response assays and did not distinguish heterogeneity in binding assays (Fisher et al, 1983). This finding contrasts with the ability of oxotremorine to distinguish subpopulations in membrane studies (Ellis and Hoss, 1980; Birdsall et al, 1980).

The above studies (Fig. 2; Fisher et al, 1983) differ qualitatively from those of Michell et al (1976) in smooth muscle and pancreas, where the response curves for PhI turnover were found to be flattened and to follow the binding curves of agonists. However, since the binding and response curves in the latter report were not generated under identical conditions, the results are difficult to interpret. Furthermore, smooth muscle may exhibit densensitization in the binding of agonists (Young, 1974), while brain does not (Birdsall et al, 1978). In parotid acinar cells, Weiss and Putney (1981) found the dose-response curve for the PhI effect to be shifted to the left, relative to the methacholine occupancy curve. This might suggest that a high-affinity subpopulation of receptors is involved or that there is a receptor reserve associated with the response. Cohen et al (1983) have reported that neurally derived

cell lines (N1E-115 and NG108-15) exhibit a robust but somewhat variable PhI response to muscarinic agonists. The response was found to desensitize within 20 min in the presence of 1 mM carbachol, a result that has not generally been reported for other systems (Weiss and Putney, 1981; Miller, 1977; Kirk and Michell, 1981; Fisher and Agranoff, 1980).

B. Cyclic nucleotides

Agonists acting at muscarinic receptors stimulate guanylate cyclase activity in intact cells and inhibit adenylate cyclase in intact cells and cell-free systems, under appropriate conditions. Presumably, these cyclic nucleotides then serve as second messengers to modulate intracellular metabolism, especially the activity of protein kinases, leading eventually to alterations in the ionic conductance of the cell membrane.

1. Guanylate cyclase

Muscarinic agonists stimulate the activity of a guanylate cyclase in brain (Hanley and Iversen, 1978; Ferendelli et al, 1970) and in cultured cells derived from neural tissue (Richelson et al, 1978). Stimulation of the cultured cell line N1E-115 can lead to a 200-fold increase in the intracellular concentration of cGMP (Matsuzawa and Nirenberg, 1975). The response is dependent on the presence of physiological levels of extracellular Ca^{2+} (Schultz et al, 1973; Hanley and Iversen, 1978). It is likely that the guanylate cyclase involved is soluble, as the membrane-bound form is inhibited by calcium (Ferendelli et al, 1976). Also, sodium azide stimulates only the membrane-bound enzyme and this

stimulation is linearly additive to that induced by oxotremorine (Hanley and Iversen, 1978). Thus, it might be assumed that muscarinic receptor activation leads to an influx of Ca^{2+} which, in turn, stimulates the activity of guanylate cyclase. However, it is doubtful that intracellular levels of Ca^{2+} ever rise to a level sufficient to stimulate the cytosolic enzyme (Deguchi et al, 1983). Indeed, Snider et al (1981) used aequorin to show that stimulation of N1E-115 cells by carbachol did not result in noticeable Ca^{2+} uptake. These findings suggest that there may be another endogeneous activator of cytosolic guanylate cyclase, and two such activators have been proposed. Snider and Richelson (1983) found that the guanylate cyclase activity of N1E-115 cells was dramatically enhanced by thrombin. Based on a combination of direct and indirect evidence, these authors (Snider and Richelson, 1983; McKinney and Richelson, 1984) have suggested that an arachidonate hydroperoxide formed by hormone-stimulated, calcium-dependent pathways may be the endogeneous activator. Deguchi et al (1983) isolated from rat brain an activator of soluble guanylate cyclase which was identified as L-arginine. D-Arginine was not effective, and the activation by (micromolar) L-arginine required the presence of low levels of Ca^{2+}. The authors postulated that Ca^{2+} is mobilized by hormone action, possibly by the ionophore action of phosphatidic acid, and that the combination of the mobilized Ca^{2+} and L-arginine would be sufficient to activate the cyclase, although it was allowed that other factors may be involved (Deguchi et al, 1983).

The kinetics of the guanylate cyclase response are complex. In brain slices and in cultured neuroblastoma (N1E-115) cells, intracellular

cGMP levels peak at 1-2 min and then decline rapidly (Hanley and Iversen, 1978; El-Fakahany and Richelson, 1980). The declining phase appears to be the result of a turning off of the stimulatory phase (McKinney and Richelson, 1984), and the action of phosphodiesterase is at least partly responsible for the reduction in cGMP levels (Matsuzawa and Nirenberg, 1975; Hanley and Iversen, 1978). The time course is very similar when phosphatidic acid (Deguchi et al, 1983) or Mn^{2+} (El-Fakahany and Richelson, 1980) is the stimulant, which suggests that the turning-off process is not mediated by the receptor itself (McKinney and Richelson, 1984). The time course of the decline of cGMP levels is very similar to that of desensitization (El-Fakahany and Richelson, 1980), which might indicate that the two processes are different manifestations of the same event.

2. Adenylate cyclase

Muscarinic agonists inhibit the activity of the enzyme, adenylate cyclase, in intact cells and cell-free preparations derived from many sources, including heart (Jakobs et al, 1979), neurally derived cell lines (Nathanson et al, 1978), and brain (Olianas et al, 1983). Hormone systems which inhibit adenylate cyclase share many of the properties of the β-adrenergic receptor-mediated activation of adenylate cyclase. The inhibitory system possesses three main components: a receptor; a GTP-binding regulatory protein; and the catalytic moiety (Cooper, 1982). The presence of GTP or its stable analogues reduces the affinities of the inhibitory agonists, and GTP is required for the inhibition of adenylate cyclase activity. Stimulatory and inhibitory hormones have both been

found to stimulate low-K_m GTPase activities in a manner that suggests that GTP hydrolysis terminates the coupling between hormone and adenylate cyclase (Jakobs et al, 1983b).

A great deal of evidence now indicates that the GTP-regulatory protein involved in the inhibitory process is different from that which leads to stimulation, and the designations N_s and N_i have been given to the stimulatory and inhibitory sites, respectively. Much of this evidence has been summarized recently (Jakobs et al, 1983b). Additionally, several very recent reports have shown that pertussis toxin selectively disrupts the coupling of inhibitory hormones to adenylate cyclase by an ADP-ribosylation, leaving the stimulatory system unaffected (Murayama and Ui, 1983; Kurose et al, 1983; Bokoch et al, 1983). Hydrolysis-resistant GTP analogues do not have completely parallel effects on stimulatory and inhibitory systems. These stable analogues lead to a persistent activation of adenylate cyclase in many cell types through interaction with N_s (Ross and Gilman, 1980). Recent evidence suggests that in the cyc^- variant of the S49 lymphoma cell line, which lacks a functional N_s, the stable analogues inhibit adenylate cyclase activated, for example, by forskolin (Jakobs, 1983a). In light of the unique aspects of inhibitory systems, care must be taken in assuming that principles determined for stimulatory systems will necessarily apply to inhibitory systems.

The muscarinic, opiate and α adrenergic receptors found on NG108-15 cells inhibit adenylate cyclase in similar manners (Sabol and Nirenberg, 1979). The α-adrenergic response has been studied in greater detail, due in large part to the fact that there is a selective pharmacology of

α receptors and that the inhibition of adenylate cyclase is mediated by the α_2 subtype (Sabol and Nirenberg, 1979; Lenox et al, 1983). Indeed, there are interesting parallels between the adrenergic system (α_1 plus α_2) and the muscarinic system. That is, α_1 receptors are associated with the turnover of the PhI and Ca^{2+} mobilization, while α_2 receptors mediate the inhibition of adenylate cyclase (Berridge, 1980). The binding of agonists to α_2 receptors is sensitive to guanyl nucleotides, while the binding to α_1 receptors is not (Lefkowitz et al, 1981). In the presence of sufficient GTP or GppNHp, Hill coefficients for the binding of agonists to α_2 receptors are raised from about 0.6 to 1.0 (Lenox et al, 1983; Tsai and Lefkowitz, 1979). The fact that guanyl nucleotides raise the Hill coefficients for muscarinic agonist binding in the brainstem and heart, but not quite to unity (Hulme et al, 1981b) may indicate the similar presence of a muscarinic subtype not regulated by GTP.

C. Ion Fluxes

Muscarinic agonists modulate the firing rate of neurons via alterations in membrane potassium conductance. Sympathetic neurons can display both inhibitory and excitatory responses (Kuba and Kobetsu, 1978). The decreases in firing rate in the heart (Vaughn-Williams, 1957; Giles and Noble, 1976) and in neurons (Ben-Ari et al, 1976; Horn and Dodd, 1981) result from increases in potassium conductance. Increases in the firing rates of central and sympathetic neurons have been attributed to decreases in potassium conductance. At least two potassium channels may be involved in excitation. One is a voltage-sensitive channel, the

M-channel, which opens as the cell depolarizes (Brown, 1984). The other channel is opened by increases in intracellular calcium levels (North and Tokimasa, 1984). Both channels serve to restore the resting membrane potential and inhibit repetitive firing. The action of muscarinic agonists is to close these channels and to increase membrane resistance, thereby rendering the neuron more sensitive to other excitatory inputs. This feature suggests that muscarinic synapses may serve a modulatory role in synaptic transmission. The slowness of muscarinic responses (Purves, 1976; Bolton, 1976) is also consistent with a modulatory role and suggests that second-messenger systems may be interposed between receptor activation and effects on conductance. Cyclic GMP has been suggested as a candidate for such a second messenger role, but there is disagreement over whether cGMP mimics the effects of muscarinic agonists on membrane conductance (Stone et al, 1975; Phillis et al, 1974; Hartzell, 1982).

D. Presynaptic Receptors

In the classical view of the neuron, receptors reside in the dendrites or soma of the post-synaptic cell. However, receptors are also found on nerve terminals, where they presumably modulate the release of transmitter; when these presynaptic receptors are sensitive to the transmitter whose release is modulated, they are considered autoreceptors (Coyle and Snyder, 1981; Starke, 1977). There is considerable evidence for the existence of muscarinic autoreceptors and presynaptic receptors. For example, muscarinic agonists inhibit the release of norepinephrine from peripheral (Fuder et al, 1982; Muscholl, 1980) and central

(Westfall, 1974) fibers. Cholinergic agonists have also been shown to modulate the release of DA in striatal slices and synaptosomes (Westfall, 1974; De Belleroche and Bradford, 1978). Szerb (1977) has shown that the potentiation by muscarinic antagonists of the release of acetylcholine from cortical and hippocampal terminals is due to the blockade of muscarinic autoreceptors. The potentiation is greatest when the concentration of acetylcholine is artificially raised by the use of acetylcholinesterase inhibitors in the assay; however, a significant effect can also be demonstrated in the absence of cholinesterase inhibition, indicating that the autoreceptors limit the release of acetylcholine under physiological conditions. The hippocampus is an ideal region in which to study muscarinic autoreceptors, because its extrinsic cholinergic innervation derives entirely from the septum, via the fornix. Lesions of the septum or fornix reduce levels of choline acetyltransferase to 5-10% of control levels and abolish muscarinic regulation of acetylcholine release (Kamiya et al, 1981; Szerb et al, 1977). However, it has been demonstrated repeatedly that such lesions do not significantly reduce the number of muscarinic receptors in the hippocampus, as would be expected if the cholinergic terminals that are lost possess muscarinic receptors (Yamamura and Snyder, 1974; Dudai and Segal, 1978; Overstreet et al, 1980; Fisher et al, 1980b; Kamiya et al, 1981). There are several possible reasons for the failure to detect autoreceptors by binding assays. First, it has been suggested that the autoreceptors may have low affinity for the ligands that are used in the binding assays (Szerb et al, 1977); however, the discrepancy in affinities may be at least partly related to the comparison of slices to

homogenates (Gilbert et al, 1979). Second, the number of autoreceptors may be very small by comparison with the number of postsynaptic receptors. Third, the reduction in autoreceptors may be obscured by an approximately equal increase in postsynaptic receptors (denervation supersensitivity). A recent study by McKinney and Coyle (1982) suggests a combination of the latter two reasons. Ablation of the nucleus basalis of Meynert led to an acute decrease (14%) in the density of cortical muscarinic receptors, with a return to normal levels by 5 wk post-lesion.

Binding studies have been more successful in confirming the existence of presynaptic muscarinic receptors on the terminals of catecholamine neurons. The adminstration of 6-hydroxydopamine, centrally and peripherally, has been shown to lead to a reduction in the binding of muscarinic ligands which is attributable to the destruction of catecholamine terminals (Sharma and Banerjee, 1978; Gurwitz et al, 1980; De Belleroche et al, 1982). However, there is not unanimous agreement, as some studies have reported transient reductions (Kato et al, 1978) or no reduction (Story et al, 1979; cf., Muscholl, 1980) under similar conditions.

IV. Relationships Among Subpopulations and Responses

Previous sections of this article have indicated that subpopulations of muscarinic receptors may be defined in several ways. These include: the subpopulations that account for the binding of most agonists; the subpopulations that have differing affinities for non-classical antagonists (especially pirenzepine) and atypical agonists (McN-A-343),

both within and among tissues; and subpopulations that may be responsible for discrete responses. A pressing question is whether the subpopulations defined by these different means are related in simple ways. Some approaches that have been applied to this problem are described below.

A. Relationships Between Subpopulations

The natural distribution of subpopulations between tissues may provide circumstantial evidence for the equivalence or non-equivalence of subpopulations. Studies of the binding of agonists have suggested that the postulation of three sites (SH, H, L) can explain the agonist binding properties of all brain regions (Birdsall et al, 1980). However, the binding of the antagonist pirenzepine suggests the presence of heterogeneity in the cortex, but not in the brainstem (Hammer et al, 1980). Since there is a fairly even distribution of SH, H, and L sites in the brainstem, it is clear that there cannot be a one-to-one relationship between the subpopulations defined according to affinities for pirenzepine and those defined by agonists. However, the availability of {^{3}H}-pirenzepine and the agonist {^{3}H}-oxotremorine-M has allowed further comparisons to be drawn within tissues. When binding assays are conducted with low concentrations of these ligands, those sites that have highest affinities are selectively labeled (Birdsall et al, 1978). Comparisons of such studies indicate that, in the cortex, the L sites possess high affinity for pirenzepine, while the SH and H sites have lower affinity for pirenzepine (Birdsall and Hulme, 1983). In view of the data described above, this relationship would not be expected to hold

for the brainstem and may indicate a greater complexity than is allowed by the SH, H, L classification scheme. It may be that there are subsets of receptors, each with a different agonist affinity, within the subpopulations defined by pirenzepine (Birdsall et al, 1984).

When muscarinic receptors are blocked by unlabeled, irreversible antagonists in the presence of low concentrations of the agonist carbachol, the subpopulation(s) with highest affinity for carbachol are selectively protected from blockade (Birdsall et al, 1978; Ellis and Hoss, 1980). Membrane preparations which have been pretreated in this manner, to enrich the proportion of sites with high affinity toward carbachol, also exhibit a greater affinity for gallamine, compared to untreated tissue (Ellis and Hoss, 1982). This implies that gallamine has a different pattern of selectivity than pirenzepine, which would be expected to display lower affinity toward such a preparation (see above). The paradigm of selective protection is particularly useful, because it does not require the preparation of new labeled ligands. Robson and Kosterlitz (1979) used a similar approach to characterize subpopulations of opiate receptors.

B. Relationships Between Subpopulations and Responses

Muscarinic receptors are linked to many different responses in many tissues and the ability to activate or antagonize selectively these responses would be of great therapeutic import. There is an obvious analogy to the benefits that have derived from the subdivision of adrenergic receptors (Weiner, 1980). The characterization of receptors may be facilitated by the study of in vitro systems, where problems of

distribution and metabolism of drugs can be most easily monitored and controlled. An ideal preparation would allow the study of multiple responses, under conditions that are amenable to the conduct of binding assays. In this way, inter-assay differences in buffer composition, tissue source, and tissue preparation would be avoided altogether and the information obtained in binding assays could be used to best advantage. There are many instances where dose-response curves generated in intact tissue from one source have been compared to binding curves generated in subcellular preparations from another tissue, often performed in separate laboratories. It is our opinion that such comparisons introduce unnecessary complexities into an already difficult task. It is known that the binding curves of agonists and antagonists can be markedly influenced by buffer conditions (Hulme et al, 1981b; Birdsall et al, 1984), tissue source (Ellis and Hoss, 1982; Birdsall and Hulme, 1983), and even the state of the tissue (i.e., intact vs. cell-free systems; Gilbert et al, 1979).

Even when such an ideal system can be utilized and metabolism or uptake of ligands minimized, a major problem remains in relating binding and response. This is that "there is no reason to suppose that there is a linear relationship between the proportion of receptors occupied by a drug and the size of response that is observed" (Stephenson, 1975). For this reason, receptors are best characterized in terms of the affinities of competitive antagonists, which, in response assays, are obtained from Schild plots (Arunlakshana and Schild, 1959; Furchgott, 1978; Tallarida, 1981). Because a competitive antagonist "acts" by preventing the access of agonists to the receptor, the affinity of such an antagonist should be